

## RESEARCH ARTICLE

## STEM CELLS AND REGENERATION

# Brain oxygen tension controls the expansion of outer subventricular zone-like basal progenitors in the developing mouse brain

Lisa Wagenführ<sup>1</sup>, Anne K. Meyer<sup>1,2</sup>, Lena Braunschweig<sup>1</sup>, Lara Marrone<sup>1,3</sup> and Alexander Storch<sup>1,3,4,\*</sup>

## ABSTRACT

The mammalian neocortex shows a conserved six-layered structure that differs between species in the total number of cortical neurons produced owing to differences in the relative abundance of distinct progenitor populations. Recent studies have identified a new class of proliferative neurogenic cells in the outer subventricular zone (OSVZ) in gyrencephalic species such as primates and ferrets. Lissencephalic brains of mice possess fewer OSVZ-like progenitor cells and these do not constitute a distinct layer. Most *in vitro* and *in vivo* studies have shown that oxygen regulates the maintenance, proliferation and differentiation of neural progenitor cells. Here we dissect the effects of fetal brain oxygen tension on neural progenitor cell activity using a novel mouse model that allows oxygen tension to be controlled within the hypoxic microenvironment in the neurogenic niche of the fetal brain *in vivo*. Indeed, maternal oxygen treatment of 10%, 21% and 75% atmospheric oxygen tension for 48 h translates into robust changes in fetal brain oxygenation. Increased oxygen tension in fetal mouse forebrain *in vivo* leads to a marked expansion of a distinct proliferative cell population, basal to the SVZ. These cells constitute a novel neurogenic cell layer, similar to the OSVZ, and contribute to corticogenesis by heading for deeper cortical layers as a part of the cortical plate.

**KEY WORDS:** Outer subventricular zone (OSVZ), Corticogenesis, Lissencephalic brain, Gyrencephalic brain, Oxygen tension, Hypoxia, Neural stem and progenitor cells

## INTRODUCTION

The development of the embryonic forebrain is an extremely well-orchestrated process controlled by intrinsic and extrinsic signals delivered to the cells in a strict temporal sequence (Carpentier et al., 2013; Dehay and Kennedy, 2007; Nieto et al., 2004; Roy et al., 2004). Disruptions of these events alter cortical growth and design because they regulate the development of progenitor cell types. During mid-gestation, the fetal cortex consists of three distinct progenitor zones that generate different subtypes of cortical neurons in a temporal order (Florio and Huttner, 2014; Molnár et al., 2006). With the onset of neurogenesis, neuroepithelial cells transform into radial glia cells

(RGCs), which are located in the ventricular zone (VZ) and form the first neurogenic layer (Haubensak et al., 2004; Kriegstein and Alvarez-Buylla, 2009). RGCs divide asymmetrically to self-renew and produce either cortical neurons directly or intermediate progenitors (IPs), which migrate into the subventricular zone (SVZ) (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001) and generate two neurons by symmetrical division (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). The third, relatively recently discovered type of cortical progenitor is located in the outer SVZ (OSVZ) and also originates from RGCs in the VZ (Fietz et al., 2010; Hansen et al., 2010; Wang et al., 2011). This cell type undergoes self-renewing divisions to generate new OSVZ progenitor cells and is able to produce IPs. Interestingly, it is known that the development of this recently identified proliferative zone is responsible for the differences in brain size among mammalian species (Kriegstein et al., 2006; Nonaka-Kinoshita et al., 2013; Stahl et al., 2013). The abundance of this progenitor population is expanded in gyrencephalic species (such as primates and ferrets) and correlates with higher complexity of cortical architecture (Fietz et al., 2010; Reillo et al., 2011; Smart et al., 2002). By contrast, lissencephalic brains of mice possess fewer OSVZ-like progenitor cells basal to the SVZ and these do not constitute a distinct cell layer, accompanied by a smaller cortical surface area (Shitamukai et al., 2011; Wang et al., 2011).

Various research groups reported that neural progenitor cells reside in a hypoxic niche in both embryonic and adult brain (Lee et al., 2001; Zhu et al., 2005). Accordingly, oxygen tension regulates the balance between neural stem cell maintenance and neural progenitor cell differentiation (Chen et al., 1999; Mohyeldin et al., 2010; Simon and Keith, 2008). These data prompted us to study whether a change of oxygen tension within the fetal brain tissue would lead to alterations in the generation of the various progenitor subtypes. The cellular response to variations in tissue oxygen tension, especially in the low-oxygen niches, is mediated by several oxygen-related molecular pathways (Bruick, 2003; Kaidi et al., 2007; Semenza, 1999). The main oxygen-sensitive transcriptional activator is hypoxia-inducible factor 1 (Hif1) (Iyer et al., 1998; Tomita et al., 2003; Zhao et al., 2008), which consists of two subunits, Hif1 $\alpha$  and Hif1 $\beta$  (also known as Arnt). Under normoxia, the Hif1 $\alpha$  subunit is continuously degraded by the ubiquitin-proteasome pathway, whereas under hypoxia the degradation pathway is circumvented. As a consequence, Hif1 $\alpha$  accumulates in the cytoplasm and translocates to the nucleus where it heterodimerizes with Hif1 $\beta$ , leading to the transcription of Hif1 target genes (Semenza, 2006; Sharp and Bernaudin, 2004).

We show here that by modulating maternal oxygenation fetal brain oxygen levels are robustly affected, subsequently leading to a modification in cortical neurogenesis: increased fetal tissue oxygen tension during mid-neurogenesis results in an accumulation of proliferative cells in regions more basal to the SVZ at the expense of

<sup>1</sup>Division of Neurodegenerative Diseases, Department of Neurology, Technische Universität Dresden, Fetscherstrasse 74, Dresden 01307, Germany. <sup>2</sup>Leibniz Institute for Solid State and Material Research, IFW Dresden, Institute for Integrative Nanosciences, Helmholtzstrasse 20, Dresden 01069, Germany. <sup>3</sup>Center for Regenerative Therapies Dresden (CRTD), Technische Universität Dresden, Fetscherstrasse 105, Dresden 01307, Germany. <sup>4</sup>German Center for Neurodegenerative Diseases (DZNE) Dresden, Arnoldstrasse 18, Dresden 01307, Germany.

\*Author for correspondence (alexander.storch@neuro.med.tu-dresden.de)

Received 9 January 2015; Accepted 15 July 2015

proliferative cells within the SVZ. Further analysis revealed that these cells contribute to corticogenesis, since an accumulation of newborn neurons in the deeper cortical layers was observed. Our findings indicate that oxygen signaling is an essential mechanism for establishing the progenitor cell identities that generate different cortical neuron subtypes.

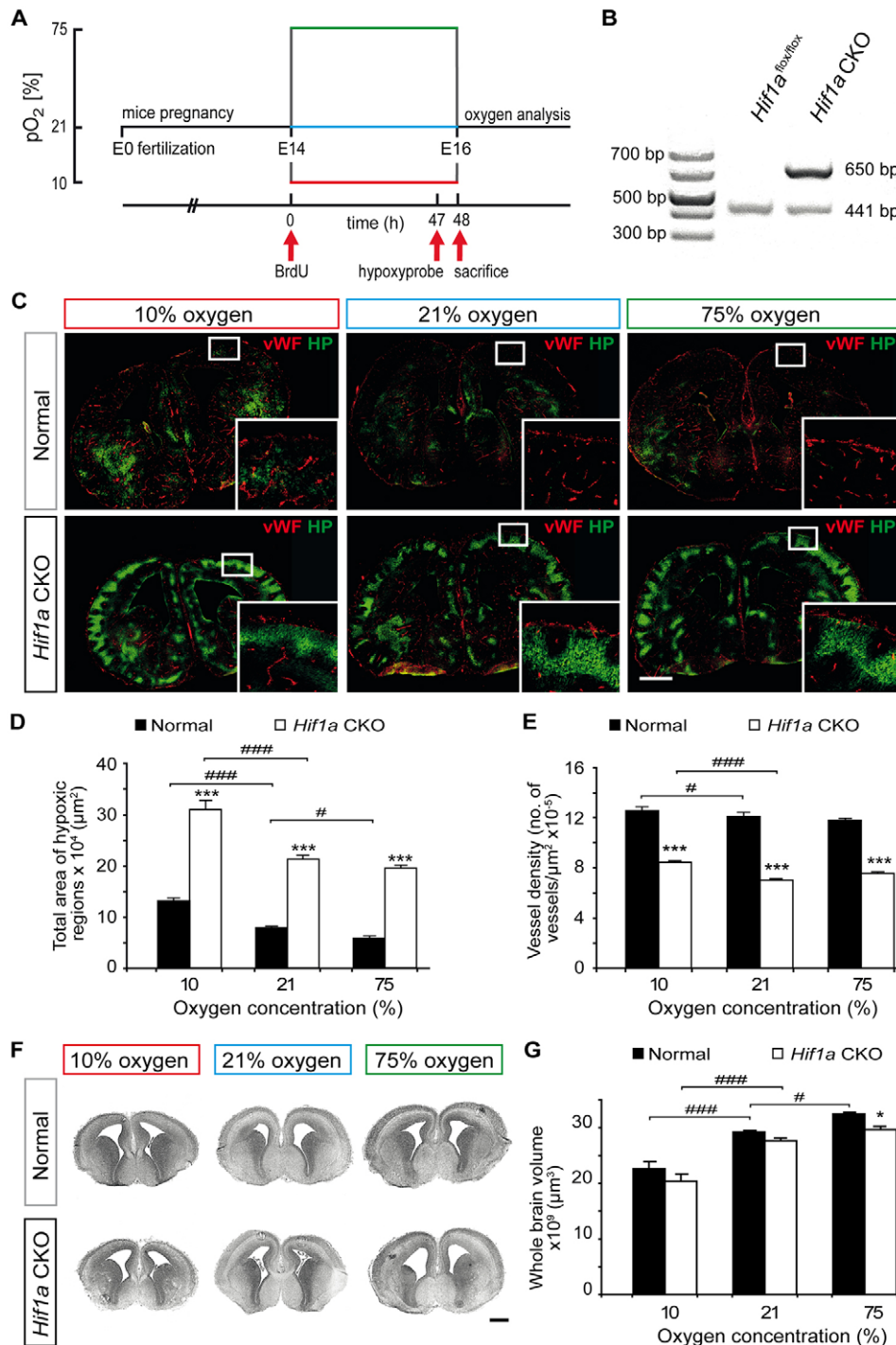
## RESULTS

### Changing maternal oxygen levels affects embryonic tissue oxygen tension and impacts vessel density and brain size

Oxygen tension in the adult mouse brain ranges from as low as 1% to 8% and is considerably lower than the inhaled ambient oxygen

tension of 21%. These oxygen variations are referred to as 'physiological normoxia' (Ivanovic, 2009). Interestingly, mammalian embryos develop in a more hypoxic microenvironment, which is crucial for regulating the development of the brain and its vasculature (Chen et al., 1999; Lee et al., 2001; Simon and Keith, 2008).

To understand the role of oxygen in cerebral cortical development during mid-neurogenesis, we first investigated the possibility of transmission of maternal hypoxia and hyperoxia to the developing brain in an *in vivo* mouse model. Embryonic day (E) 14 timed-pregnant mice were kept under 10%, 21% and 75% oxygen for 48 h in an airtight plexiglass chamber (Fig. 1A). Normoxic mice were incubated in room air. Studying oxygen tension *in utero* is highly



**Fig. 1. Availability of oxygen in fetal mouse forebrain after changes in maternal oxygenation.**

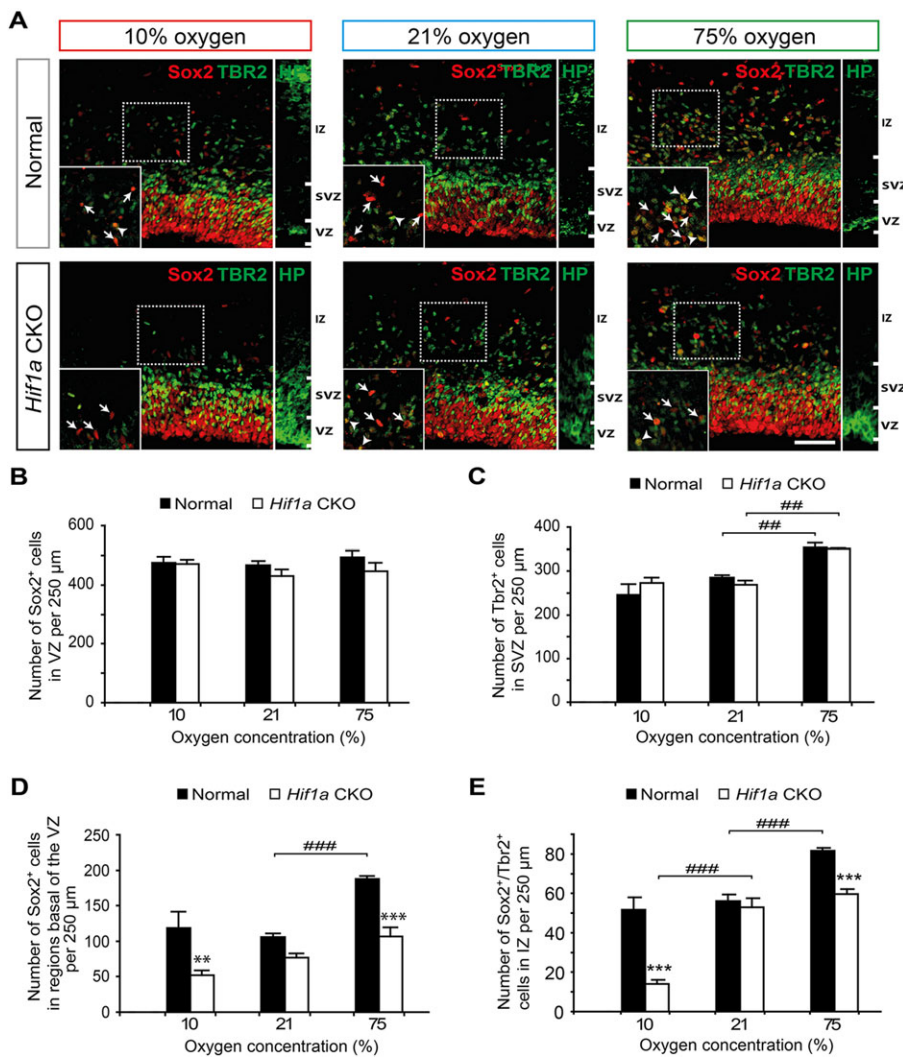
(A) Experimental setup to study oxygen effects on fetal brain development. Pregnant mice (E14) were exposed to different oxygen concentrations (10%, 21%, 75%) for 48 h. To assess hypoxic regions in the fetal brain, a single pimonidazole hydrochloride (hypoxyprobe) injection was administered after 47 h and embryonic brains were dissected 1 h later. For the birth-dating study, pregnant mice were injected with a single dose of BrdU before oxygen treatment. (B) PCR analysis of genomic DNA isolated from tails of normal and *Hif1a* transgenic mice. Primers 2 and 3 generate a 441 bp band for the floxed allele of the *Hif1a<sup>fllox/fllox</sup>* mouse (denoted as normal, see Materials and Methods). Estimation of Cre-mediated recombination in the tissue of *Hif1a* CKO mice employed specific primers that generate a 650 bp band. (C) Hypoxyprobe (HP, green) and von Willebrand factor (vWF) vessel (red) staining of coronal sections throughout forebrains of normal and *Hif1a* CKO embryos at E16. Boxed areas are shown at higher magnification in insets. (D) Quantitative analysis of the hypoxic level in the forebrain after oxygen exposure in normal and mutant samples. (E) Quantification of vessel density in normal and mutant forebrain after maternal hypoxia/hyperoxia compared with normoxia. (F) Histological analysis of normal and *Hif1a* mutant brains after maternal hypoxia/hyperoxia for 48 h. Coronal forebrain sections stained with Hematoxylin and Eosin revealed an increased brain size in the hyperoxic littermates. (G) Quantitative assessment of brain volume using stereological techniques at E16. Data are mean ± s.e.m.  $n=4-8$ . # $P<0.05$ , ### $P<0.001$ , \* $P<0.05$ , \*\*\* $P<0.001$ , compared with control (two-way ANOVA, Bonferroni-adjusted). Asterisks indicate statistical significance between different genotypes for the same oxygen concentration, whereas hash marks indicate statistical significance between different oxygen concentrations for the same genotype. For full statistics for D, E and G see supplementary material Tables S1, S2 and S3, respectively. Scale bars: 500 μm.

challenging and a wide range of techniques have been established for measuring oxygen tensions (Davies and Brink, 1942; Seylaz and Pinard, 1978). In our study, we used immunohistochemically detectable pimonidazole hydrochloride, a non-invasive hypoxia marker highly suited for use in smaller animals (Raleigh et al., 1987). Using this tool we were able to analyze the tissue distribution of hypoxic regions on a subregional level and obtained information about correlations between tissue oxygen tension and subsequent cellular responses (Figs 1 and 2). Oxygen levels are known to act as regulators of stem cell maintenance, proliferation and differentiation through the activation of several oxygen-sensitive molecular pathways, among which *Hif1 $\alpha$*  appears to be dominant, although its role in cortical progenitors has not been thoroughly investigated. To evaluate whether *Hif1 $\alpha$*  is necessary to translate oxygen changes into cellular responses, we conditionally knocked out (CKO) *Hif1 $\alpha$*  exons 13 to 15 in neuroprogenitors using nestin promoter-driven expression of Cre recombinase (Tomita et al., 2003) (Fig. 1B).

Confocal analysis of normal, normoxic coronal forebrain sections obtained from E16 embryos revealed weakly detectable pimonidazole adducts throughout the forebrain (Fig. 1C,D). As expected, the percentage of hypoxic tissue within the normal fetal brain was increased by maternal hypoxia (Fig. 1C,D). Accordingly, a decline of hypoxic tissue was observed in hyperoxic normal fetal brains (Fig. 1C,D). A detailed analysis of the hypoxic level in fetal

*Hif1 $\alpha$*  CKO mouse brains revealed abundant hypoxic tissue in all forebrain areas but particularly where the vessels starts to sprout and invade into the cortical plate (Ma et al., 2013). *Hif1 $\alpha$*  regulates angiogenesis during embryonic brain development (Hashimoto and Shibasaki, 2015) and, indeed, angiogenesis was disrupted in the knockout mice and thus a normal oxygen supply could not be ensured (Fig. 1E). However, maternal hypoxia slightly promoted vessel growth to compensate the oxygen undersupply in the *Hif1 $\alpha$*  CKO brain, indicating that other oxygen-dependent pathways compensated for the loss of *Hif1 $\alpha$*  (Fig. 1C,E). Mammalian embryogenesis occurs in a very low oxygen environment until E11 (Lee et al., 2001). Thereafter, blood vessels are generated throughout the fetal brain to increase the oxygen supply. Inactivation of *Hif1 $\alpha$*  in all neuroprogenitor cells during brain development led to an apparent loss of vessels throughout the forebrain at E14 and thus to consistent decreased tissue oxygenation (supplementary material Fig. S1A,B).

Compared with normoxic brains, histological sections revealed a significantly reduced fetal brain volume of hypoxia-treated embryos, whereas hyperoxia provoked a significant increase in brain volume (Fig. 1F,G). *Hif1 $\alpha$*  CKO in progenitors does not apparently affect the development of the normoxic fetal forebrain, as coronal sections of these brains exhibited similar growth and cytoarchitecture in both genotypes at various developmental stages



**Fig. 2. Maternal hyperoxia leads to an expansion of Sox2<sup>+</sup> and Tbr2<sup>+</sup> cells basal to the SVZ, constituting a new neurogenic layer in E16 mouse dorsal telencephalon.** (A) Coronal sections of the developing cortex of oxygen-treated normal and *Hif1 $\alpha$*  CKO mice at E16 were analyzed using Sox2 (red), a marker for apical progenitors, and Tbr2 (green), a marker for IPs. Images are presented with hypoxyprobe (HP) staining for oxygen reference. Boxed areas are shown at higher magnification in insets. Arrowheads indicate cells co-expressing Sox2 and Tbr2 within the OSVZ, while arrows indicate cells that express Sox2 alone. Scale bar: 50 μm. (B,C) Quantification of the number of Sox2<sup>+</sup> and Tbr2<sup>+</sup> cells within the VZ and SVZ, respectively. (D) Quantification of the number of Sox2<sup>+</sup> cells outside and more basal to the VZ (SVZ, IZ). (E) Progenitor cells, which are double positive for Sox2 and Tbr2, were counted in hypoxic, normoxic and hyperoxic external to the SVZ of E16 normal and *Hif1 $\alpha$*  CKO forebrains. Data are mean±s.e.m.  $n=4$ . ### $P<0.01$ , #### $P<0.001$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ , compared with control (two-way ANOVA, Bonferroni-adjusted). Asterisks indicate statistical significance between different genotypes for the same oxygen concentration, whereas hash marks indicate statistical significance between different oxygen concentrations for the same genotype. For full statistics for B-E see supplementary material Tables S4-S7, respectively.



(Fig. 1F,G; supplementary material Fig. S1C), indicating no prominent role of the *Hif1 $\alpha$*  pathway in embryonic brain development.

Together, maternal oxygen treatment translates into robust changes in the tissue oxygenation of fetal brain in mice, as a representative small animal model, and this results in alterations of whole brain volume. Moreover, *Hif1 $\alpha$*  acts as a stimulator for angiogenesis but does not appear to be involved in cellular processes of cortical neurogenesis.

### Oxygen regulates the number of Sox2<sup>+</sup> and Tbr2<sup>+</sup> cells in regions basal to the SVZ

We next hypothesized that altered tissue oxygen tension within the neurogenic zones of the fetal brain might control the precise pattern of cell division, differentiation and survival of neuroprogenitors. First, we ascertained whether altered oxygen tension within the neurogenic niches affects the number of progenitors in the forebrain. The developing cortex consists of different proliferative populations. The first population comprises dividing Sox2<sup>+</sup> RGCs at the ventricular surface of the VZ (Haubensak et al., 2004; Hutton and Pevny, 2011; Kriegstein and Alvarez-Buylla, 2009; Malatesta et al., 2000). The second class of neural progenitor cells, which originate from RGC mitoses, are referred to as Tbr2<sup>+</sup> IPs (Tbr2 is also known as Eomes) and form the SVZ (Bulfone et al., 1999; Englund et al., 2005; Gal et al., 2006; Kriegstein et al., 2006; Miyata et al., 2004). A third proliferative zone, referred to as the OSVZ, has been identified in the developing human embryonic neocortex; it is generated by RGCs and is located directly above the Tbr2<sup>+</sup> SVZ (Fietz et al., 2010; Hansen et al., 2010). Recently, it was shown that a small number of these OSVZ progenitor cells is also present in the rodent intermediate zone (IZ), but they do not form a distinct neurogenic layer in contrast to the OSVZ in primates (Shitamukai et al., 2011; Wang et al., 2011). The three progenitor populations produce different cortical subtypes in a controlled temporal order (Lukaszewicz et al., 2005; Parnavelas, 2000; Vasistha et al., 2014). Inspection of coronal sections of fetal mouse forebrains revealed no difference in the radial thickness of VZ or SVZ by hypoxic/hyperoxic treatment or *Hif1 $\alpha$*  CKO (Fig. 2A). Surprisingly, we detected an apparent accumulation of Sox2<sup>+</sup> cells (arrows in Fig. 2A) in hyperoxic forebrains that partially co-express Tbr2 (arrowheads in Fig. 2A) in the superficial SVZ and form a separate layer. Using hypoxyprobe-based estimation of tissue oxygen tension, we observed that the oxygen level of the IZ, in particular, varied in the normal brains in response to hypoxic/hyperoxic treatment and seems to correlate with the cellular response of these cells (insets in Fig. 2A). The VZ remains hypoxic and does not appear to be susceptible to manipulation by maternal oxygen treatments.

We next quantified the number of Sox2<sup>+</sup> and Tbr2<sup>+</sup> cells in their ‘regular’ regions and Sox2<sup>+</sup> cells outside of the supposed VZ (Fig. 2B–D). Based on this quantification, Insolera and co-workers showed that the accumulation of Tbr2<sup>+</sup> and Pax6<sup>+</sup> (another radial glia marker) cells within the IZ induced by various interventions is caused by changes in migration and accompanied by a selective loss of these cell types within the VZ and SVZ (Insolera et al., 2014). By contrast, we observed that the number of Sox2<sup>+</sup> cells within the VZ of the hyperoxic forebrain was indistinguishable from that of normoxic and hypoxic forebrains. Also, inactivation of *Hif1 $\alpha$*  in neuroprogenitors did not affect the apical progenitor population within the VZ (Fig. 2B). Interestingly, the Tbr2<sup>+</sup> IP population in the SVZ was significantly expanded in normal and mutant hyperoxic cortex (Fig. 2C). Moreover, there were more Sox2<sup>+</sup>

cells located in the regions basal to the VZ in hyperoxic compared with normoxic cortex (Fig. 2D). The number of Sox2<sup>+</sup>/Tbr2<sup>+</sup> cells in regions basal to the SVZ was also significantly elevated (Fig. 2E). Indeed, these Sox2<sup>+</sup>/Tbr2<sup>+</sup> cells were obviously reduced in hypoxic *Hif1 $\alpha$*  CKO cortex compared with normal cortex (Fig. 2E).

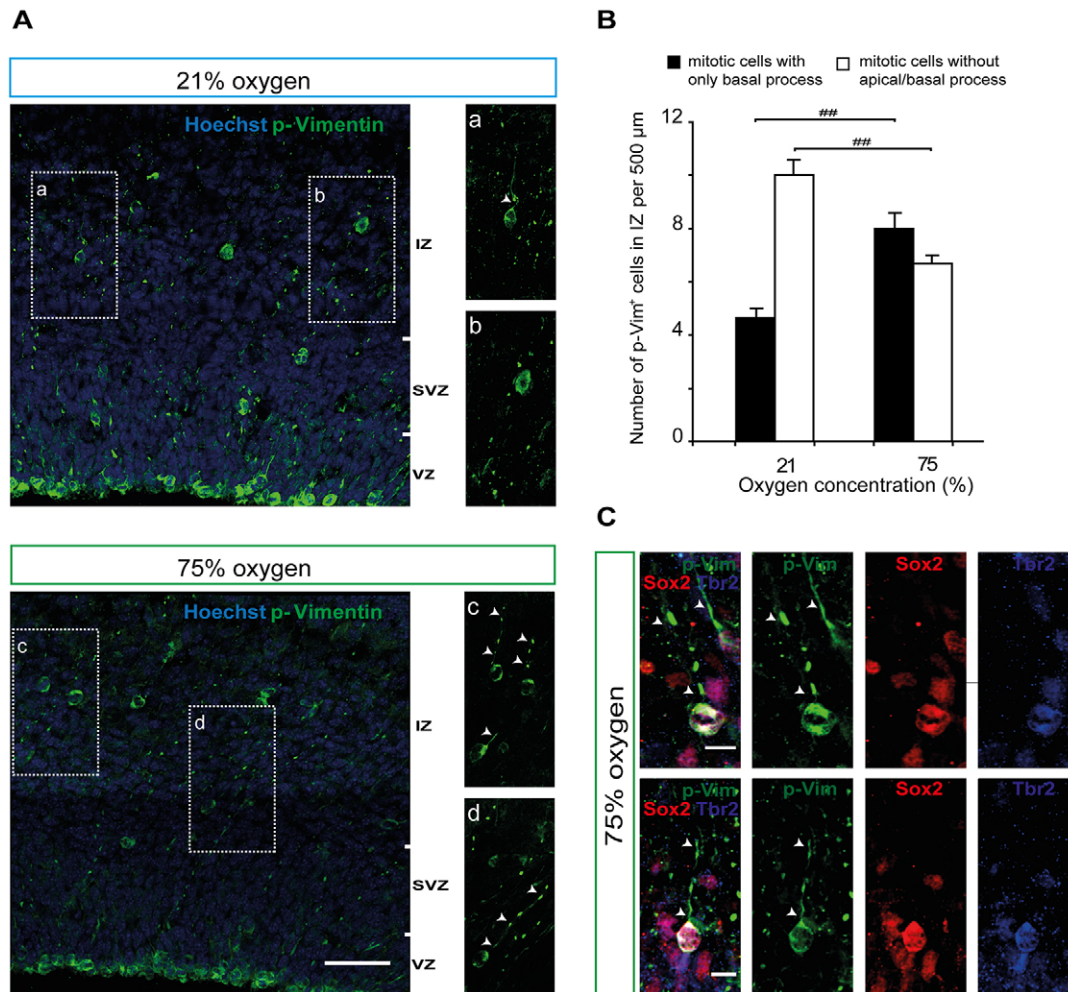
Programmed cell death is frequent in neural progenitors during brain development and is involved in regulating cerebral cortical size (Blaschke et al., 1996; Haydar et al., 1999). Cell death analysis using TUNEL staining revealed that the distribution of apoptotic cells was uniformly spread throughout the three proliferative zones in the normoxic cortex. Interestingly, hyperoxia-treated animals showed a significant reduction in TUNEL<sup>+</sup> cell numbers as compared with normoxic animals (supplementary material Fig. S2A,B, Table S11). Thus, short-term maternal hyperoxia is beneficial for the progenitors within the various neurogenic niches, without any increase in the apoptosis rate. Tissue with a reduced oxygen supply due to hypoxic treatment exhibited significantly elevated, and more disorganized, apoptosis within the VZ, SVZ and IZ than normoxic samples (supplementary material Fig. S2A,B), accompanied by a decrease in fetal brain volume.

These results demonstrate that maternal hyperoxia causes an expansion of Sox2 and Tbr2 single- and double-positive progenitors basal to the SVZ without depleting the normal population of VZ and SVZ progenitors. Moreover, the emergence of this cell layer is specifically regulated by oxygen tension.

### Increased number of proliferative OSVZ-like cells in hyperoxic fetal cortex

Characterization of the various neural progenitor cell types is possible on the basis of morphology. A hallmark of OSVZ progenitors is their radial glia-like morphology but without an apical process, and they are therefore easily distinguishable from RGCs (with apical and basal processes) and IPs (without apical and basal processes) (Fietz et al., 2010; Hansen et al., 2010). We examined the presence of basal processes of mitotic progenitor cells in the IZ using phosphovimentin (p-Vim) staining as a specific marker (Kamei et al., 1998). We observed more p-Vim<sup>+</sup> cells with only basal processes in the IZ of the hyperoxic cortex than in normoxic brains, indicating that these cells are OSVZ-like cells and not delocalized RGCs or IPs (Fig. 3A,B). The OSVZ-like cell population in the rodent brain shows sustained expression of the radial glia marker Sox2, but not Tbr2, and is therefore distinguishable from Tbr2<sup>+</sup>/Sox2<sup>-</sup> IPs (Wang et al., 2011). They divide asymmetrically to self-renew and produce IPs by gradually downregulating the radial glia marker Sox2 and upregulating Tbr2. For better classification of the monopolar cells, we stained the forebrain sections additionally for Sox2 and Tbr2 (Fig. 3C). Surprisingly, p-Vim<sup>+</sup> cells with basal process were also Sox2<sup>+</sup>/Tbr2<sup>+</sup>, which means that these cells resembled RGCs and IPs, comparable to the primate OSVZ progenitor population (Betizeau et al., 2013).

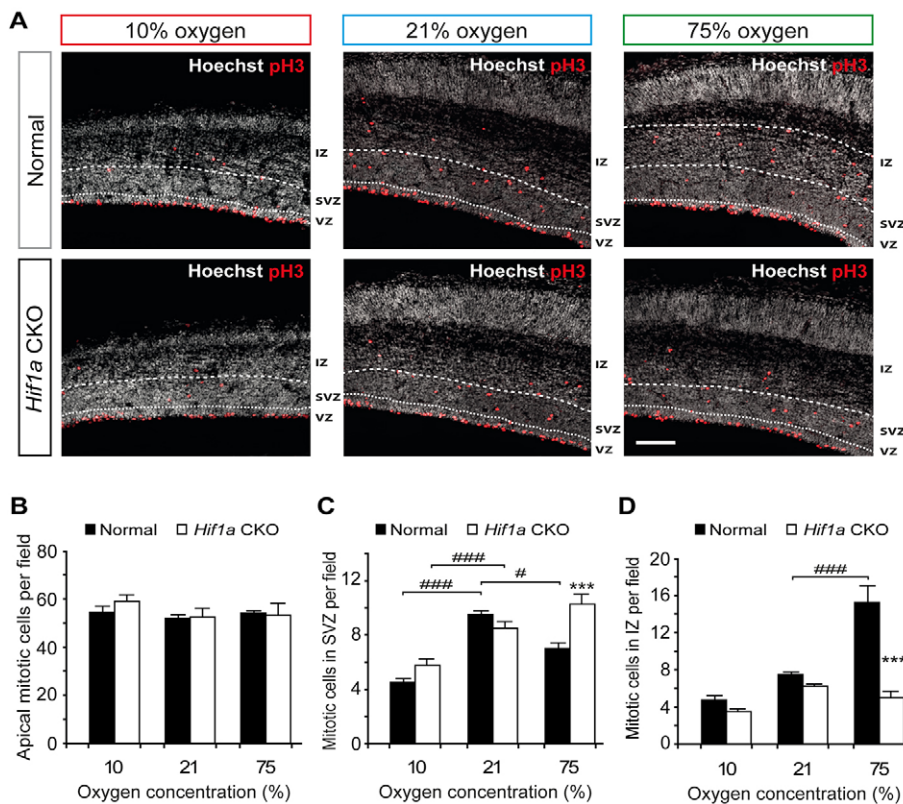
The neuroprogenitor expansion within the IZ does not occur at the expense of progenitors in the VZ or SVZ of the hyperoxic cortex. To examine whether this accumulation of Sox2<sup>+</sup> progenitor cells is based on increased self-renewing divisions of existing cells within the IZ or is due to an increase in apical and basal mitoses, we stained E16 coronal sections for phospho-histone H3 (pH3) to label mitotic cells in VZ, SVZ and IZ (Fig. 4A). The abundance of mitotic apical progenitor cells along the VZ was not altered in the differently oxygenated brains, suggesting normal development of the VZ and SVZ, which are generated by asymmetric divisions of the RGCs (Fig. 4B). Hypoxia-treated animals of both genotypes showed a decrease in pH3<sup>+</sup> basal progenitor cells in the SVZ



**Fig. 3. An increased number of mitotic progenitor cells show long basal processes in the hyperoxic forebrain.** (A) Representative images of E16 normoxic and hyperoxic brain sections stained for phospho-vimentin (p-Vim; green) to label all progenitors in mitosis. Higher magnification images (a-d) show OSVZ-like cells with the typical basal process in both oxygen conditions. Arrowheads indicate p-Vim<sup>+</sup> basal processes. (B) The number of p-Vim<sup>+</sup> cells with or without a basal process located in regions basal to the SVZ in E16 normoxic and hyperoxic mouse brain. Statistical analysis revealed significant differences between both oxygen tensions. Data are mean $\pm$ s.e.m.  $n=4$ .  $^{***}P<0.01$  (unpaired Student's *t*-test, Bonferroni-adjusted). (C) Representative images of normal hyperoxic forebrain sections at E16 showing OSVZ-like p-Vim<sup>+</sup> cells, which have a basal but no apical process, stained for the RGC marker Sox2 and the IP marker Tbr2. Arrowheads indicate p-Vim<sup>+</sup> basal processes. Scale bars: 50  $\mu$ m in A; 10  $\mu$ m in C.

(Fig. 4C), suggesting that neuroprogenitors of the CKO brains react similarly to maternal hypoxia despite inactivation of Hif1 $\alpha$ . However, within the IZ – where an accumulation of Sox2<sup>+</sup> cells had been noticed previously – more mitotic cells were observed in hyperoxic brains (Fig. 4A,D), along with a reduced number of proliferative cells within the SVZ (Fig. 4C). This indicates that progenitor accumulation within this zone is mediated by an intrinsic increase of proliferation. However, the total number of dividing cells within the SVZ/IZ of the hyperoxic CKO system was similar to that in the normoxic control brain (supplementary material Fig. S3). These effects demonstrate that 48 h of hyperoxia were unable to change tissue oxygen tension, owing to a lack of blood vessels throughout the fetal brain caused by *Hif1a* CKO. Since the inactivation of Hif1 $\alpha$  does not result in differences in neuroprogenitor properties under normoxic conditions, we infer that Hif1 $\alpha$  is fundamental for vascularization but is not directly responsible for variations in neuronal proliferation. Oxygen is the main regulator here, and the differences between mutant and control brains after oxygen treatment seem to be primarily induced by variations in oxygen tension.

To evaluate whether the observed neurogenic response to altered oxygen availability is specific to later stages of corticogenesis or also affects progenitor activity at earlier developmental stages, we investigated the formation of the SVZ after 48 h incubation at 75% atmospheric oxygen tension in E12 forebrains (supplementary material Fig. S4A). We could not detect any increase in cell proliferation (Tbr2<sup>+</sup>/pH3<sup>+</sup> cells) within the SVZ, nor any alteration in the number of intermediate Tbr2<sup>+</sup> progenitors upon hyperoxia (supplementary material Fig. S4B-D, Tables S12 and S13). We observed a similar pattern of hypoxic tissue as in E16 brains, as measured with the hypoxyprobe-based method (supplementary material Fig. S4B), but we did not detect any changes in tissue oxygen tension in the fetal brain after maternal hyperoxic treatment with 75% atmospheric oxygen for 48 h. This result supported the idea that, owing to the low vascularization, tissue oxygenation of the fetal brain is not sufficiently susceptible to manipulation at this early developmental stage. Therefore, the question of whether oxygen has temporally specific effects on the development of the various neurogenic layers remains open.



**Fig. 4. Distribution of mitotic cells in prenatal forebrain after maternal hypoxia, normoxia or hyperoxia.** (A) Fluorescent micrographs of normal and *Hif1a* CKO coronal sections at E16 showed phospho-histone H3 (pH3; red)-positive cells along the VZ, SVZ and IZ. Nuclei are stained with Hoechst (white). Scale bar: 100  $\mu$ m. (B-D) Quantification of mitotic cells within the VZ, SVZ and IZ. Data are mean  $\pm$  s.e.m.  $n=4$ . # $P<0.05$ , ### $P<0.001$ , \*\*\* $P<0.001$ , compared with the normal genotype (control) (two-way ANOVA, Bonferroni-adjusted). Asterisks indicate statistical significance between different genotypes for the same oxygen concentration, whereas hash marks indicate statistical significance between different oxygen concentrations for the same genotype. For full statistics for B-D see supplementary material Tables S8-S10, respectively.

Together, neuroprogenitor expansion within the hyperoxic IZ is generated by increased self-renewing divisions of the existing progenitors. Moreover, a higher proportion of these proliferative cells exhibit morphological properties similar to those of OSVZ progenitors.

### Fetal brain oxygenation is essential for the generation of cortical subtypes

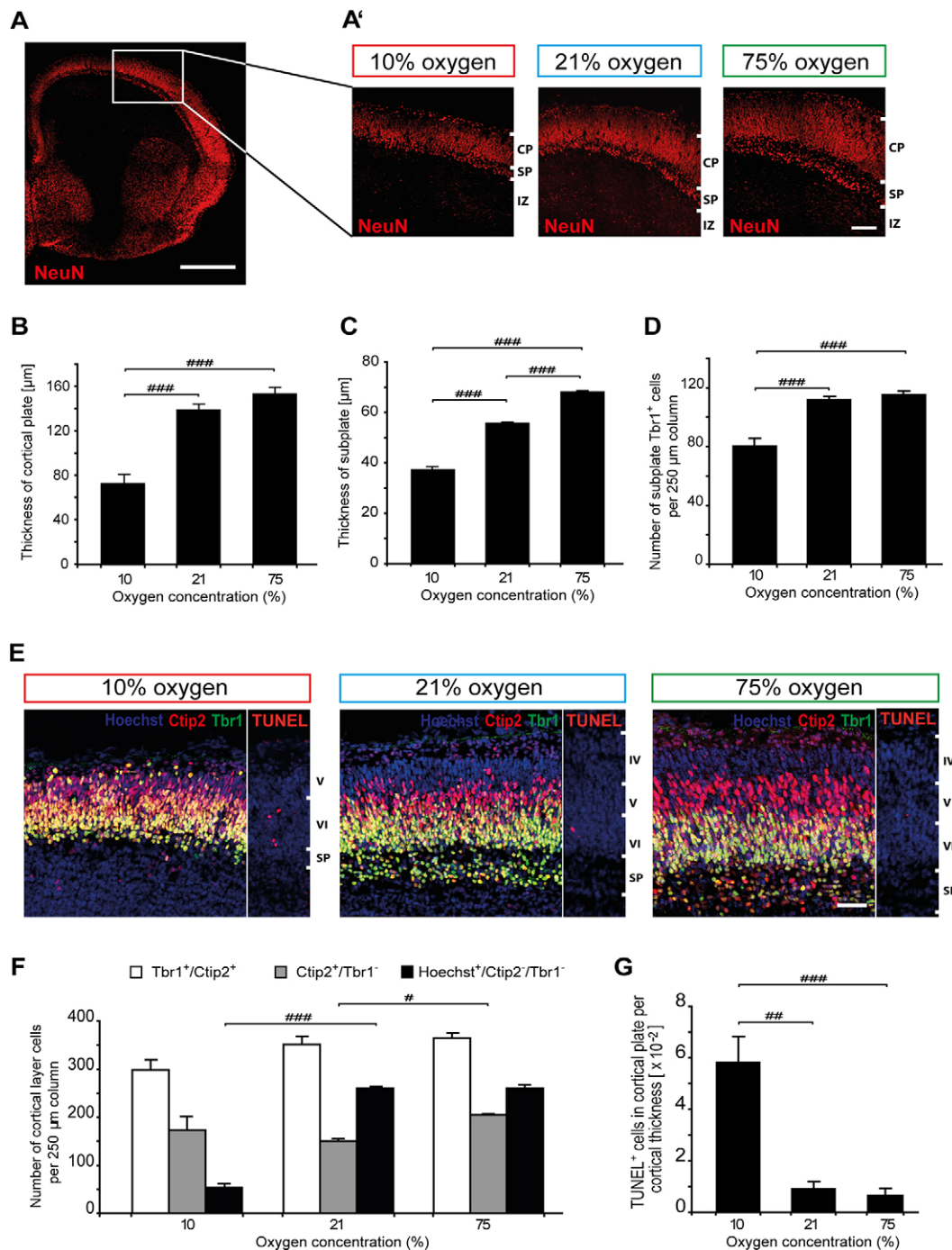
During embryonic brain development, neural stem and progenitor cells generate cortical neurons in a distinct temporal order (Molyneaux et al., 2007). The differences in progenitor abundance, proliferative capacities and neurogenesis among mammalian species are presumably the reason for variations in brain size and surface area (Borrell and Reillo, 2012; Chenn and Walsh, 2002; Fish et al., 2008). After determining the elevated proliferation of OSVZ-like progenitors upon higher oxygen supply, we further investigated whether these newly generated cells of hyperoxic brains contribute to corticogenesis. We examined cortical plate (CP) morphology at E16 using the neuronal marker NeuN (Rbfox3) (Fig. 5A). Hypoxia-treated brains revealed an extremely thin cortex with morphologically altered cortical structure compared with normal or hyperoxic cortices (Fig. 5A,A',B). Moreover, the well-defined subplate (SP) in normoxic and hyperoxic brains was considerably thinner and poorly defined in hypoxic brains. The SP and CP boundary layer in hypoxic cortices was less distinct (Fig. 5A').

During early cortical layer development (E11), the earliest born neurons are primarily destined for the preplate (PP) located above the existing proliferative zones (VZ and SVZ) (Marin-Padilla, 1978). Later-born neurons split this layer into SP and marginal zone (MZ) and form the CP in between these layers (Molnár et al., 2006; Price et al., 1997). Quantification showed that SP thickness was reduced in hypoxic animals at E16 (Fig. 5C). Interestingly, maternal hyperoxia led to an increase in SP thickness, although the generation of neuronal SP cells should have been complete at the

time of the oxygen challenge (Price et al., 1997; Sheppard and Pearlman, 1997). We thus evaluated whether more SP cells were generated during maternal hyperoxia or whether we only see an expansion of the existing SP cells. The layer-specific marker *Tbr1* is mainly expressed in the SP and layer VI (Hevner et al., 2001). The number of *Tbr1*<sup>+</sup> cells in the SP of hyperoxic cortex was comparable to that in the normoxic brain. Only in the hypoxic SP were significantly fewer *Tbr1*<sup>+</sup> cells observed (Fig. 5D).

The CP consists of six conserved layers in mammals, which differ in size between species (Molnár et al., 2006). Elevated neurogenesis for enlargement of the CP is dependent on the absolute number of progenitors involved. The SVZ/OSVZ is the main regulator for neocortical expansion since this is the only region that expands over the course of neurogenesis in gyrencephalic species, and particularly extensively in primates (Nonaka-Kinoshita et al., 2013; Smart et al., 2002; Stahl et al., 2013). During cortical development, early-born neurons are generated by progenitors in the VZ at E11.5-13.5 and form deeper layers, including SP, layer VI and V (Haubensak et al., 2004; Price et al., 1997). In the late part of neurogenesis (E14.5-18.5), neurons of the upper layers IV to II are born (Molyneaux et al., 2007). The bulk of cortical neurons are generated from SVZ progenitors, which migrate into defined layers of the CP according to an 'inside-out' pattern (Kowalczyk et al., 2009; Vasistha et al., 2014). To further analyze cortical lamination in response to oxygen tension we performed immunohistochemical staining to characterize the developing neocortical layers. *Tbr1* (SP and layer VI) (Hevner et al., 2001) and *Ctip2* (*Bcl11b*) (layer VI and V) (Arlotta et al., 2005) were used to estimate deep cortical layering. All brain samples exhibited a layered expression of these markers, suggesting an overall normally layered neocortical organization and generally normal migration (Fig. 4E). Exclusively, the hypoxic fetal forebrains possessed a thinner CP and fewer cortical neurons. At the developmental stage investigated, all *Tbr1*<sup>+</sup> cells of the CP





**Fig. 5. Fetal brain oxygenation controls cortical thickness and cortical layering.** (A) Immunohistochemistry for the neuronal marker NeuN (red) in normoxic forebrain at E16. (A') Higher magnification of the area represented by the box in A, showing NeuN staining of representative coronal sections of oxygen-treated brains from E16 embryos. (B) Quantitative analysis of CP thickness following different oxygen treatments. Radial thickness of the cortex was measured as the distance from the SP/CP boundary to pia in 100 μm intervals. (C) Radial thickness of the SP was quantified in the forebrains after oxygen exposure at E16. (D) Quantification of the number of Tbr1<sup>+</sup> cells in the SP of the oxygen-treated brains. (B-D) There were statistically significant differences between the atmospheric oxygen tensions ( $F$ -value=73.7, 333.7 and 38.6, respectively, for B-D). (E) Corresponding coronal sections from hypoxic, normoxic and hyperoxic brains were immunostained for Tbr1 (green) to mark deep layer VI neurons and for Ctip2 (red), which labels layer V neurons. Insets show TUNEL staining of CP sections after maternal hypoxia/hyperoxia or normoxia. (F) Quantification of the number of Tbr1<sup>+</sup>/Ctip2<sup>+</sup>, single Ctip2<sup>+</sup> and Tbr1<sup>-</sup>/Ctip2<sup>-</sup>/Hoechst<sup>+</sup> cells in 250 μm columns. There were significant differences between atmospheric oxygen tensions for the single Ctip2<sup>+</sup> population ( $P=0.016$ ,  $F$ -value=9.1) and Tbr1<sup>-</sup>/Ctip2<sup>-</sup>/Hoechst<sup>+</sup> cell population ( $P<0.001$ ,  $F$ -value=365.4). (G) Quantification of TUNEL<sup>+</sup> nuclei within the CP showed increased cell death in hypoxic forebrains. There were significant differences between atmospheric oxygen tensions ( $P=0.002$ ,  $F$ -value=19.8). Data are mean±s.e.m.  $n=4$ . # $P<0.05$ , ## $P<0.01$ , ### $P<0.001$  (one-way ANOVA, Bonferroni-adjusted). Scale bars: 500 μm in A; 100 μm in A'; 50 μm in E.

co-expressed Ctip2 and it was thus difficult to distinguish layer VI from layer V. Therefore, the numbers of Tbr1<sup>+</sup>/Ctip2<sup>+</sup>, single Ctip2<sup>+</sup> and Hoechst<sup>+</sup>/Tbr1<sup>-</sup>/Ctip2<sup>-</sup> cells were determined (Fig. 4F). Fewer

Ctip2<sup>-</sup>/Tbr1<sup>-</sup> upper layer neurons were generated during maternal hypoxia (Fig. 5E,F; supplementary material Fig. S5A,B), presumably owing to the aforementioned reduced SVZ/OSVZ

proliferation and increased apoptosis (Fig. 4C and Fig. 5E,G). According to these findings, the cortical layer cell type  $Tbr1^{-}/Ctip2^{+}$  represents the bulk of cortical cells within the hypoxic cortex (supplementary material Fig. S5A,B,D). The absolute number and percentage of  $Tbr1^{+}/Ctip2^{+}$  cells of layer VI were unchanged in hyperoxic as compared with normoxic samples (Fig. 5F; supplementary material Fig. S5C), whereas single  $Ctip2^{+}$  neurons were significantly increased by hyperoxia (Fig. 5F; supplementary material Fig. S5A,B,D).

In summary, maternal hyperoxia led to an expansion of  $Tbr1^{-}/Ctip2^{+}$  neurons in the fetal brain. Lower tissue oxygen concentration did not affect the inside-out pattern of neurogenesis, but restricted generation of the upper layers and the survival of cortical neurons.

### Oxygen controls the ratio between lower and upper layers

Cortical subtype specification is believed to be controlled on the one hand by several transcriptional factors as well as extrinsic signals and, on the other hand, by a subset of fate-restricted progenitors (Alcamo et al., 2008; Franco et al., 2012; Frantz and McConnell, 1996; Shen et al., 2006). To further investigate the expansion of deep cortical layers in hyperoxia, we sought to evaluate cortical neurogenesis and fate determination using a birth-dating study. Pregnant dams were injected with BrdU at E14 before oxygen treatment (Fig. 1A). At this developmental stage the deeper layer neurons are already born and show integration at their respective regions (SP and layer VI), while late-born neurons for upper layers are starting to be generated. After 48 h of oxygen exposure, fetal brains were collected and stained for BrdU and NeuN. The distribution of labeled cells in the CP of hypoxic and normoxic samples was equivalent and the generated neurons showed laminar positioning within the uppermost layers (Fig. 6A). The abundance of labeled cells born at E14 and present at E16 was ascertained within the CP by scoring from pia to the SP/CP boundary in 200  $\mu\text{m}$  counting fields (Fig. 6B). Intriguingly, in the hyperoxic cortical tissue, the majority of  $\text{BrdU}^{+}$  neurons were located in the lower layers (Fig. 6A,B) and not, as expected, within the upper cortical zone, where only a small fraction of  $\text{BrdU}^{+}$  neurons was observed (Fig. 6C).

To ascertain whether the  $\text{BrdU}^{+}$  cells are integrated or migrated further to their proper destination, we stained the normoxic and hyperoxic sections for  $Ctip2$  (representative for deeper layers) and  $Cux1$  (representative for upper layers) to determine the real number of integrated neurons within these zones (Fig. 6D). The number of  $\text{BrdU}^{+}/Ctip2^{+}$  cells within the SP of hyperoxic brains was similar to that in normoxic brains (Fig. 6E), which is in agreement with the data reported in Fig. 5D. Indeed, we discovered significantly more integrated cells in the  $Ctip2^{+}$  zone of hyperoxic brains and fewer  $\text{BrdU}^{+}/Cux1^{+}$  cells in the upper layer (Fig. 6F,G). The total number of integrated cortical neurons, however, was the same in hyperoxic and normoxic brains, but we found an apparent decrease in the number of  $\text{BrdU}^{+}$  cells within the CP of hyperoxic brains (Fig. 6B). This indicates that in the normoxic condition more migrated cells reside in the CP, and an increase in fetal oxygen tension led either to retarded migration or less cortical output due to the simultaneous accumulation of neuroprogenitors within the IZ.

Together, in response to increased cortical oxygen tension, cell proliferation within the IZ increases, whereas SVZ progenitor proliferation decreases, resulting in diminished generation of upper cortical layer neurons, with more newborn neurons heading for deeper layers.

### DISCUSSION

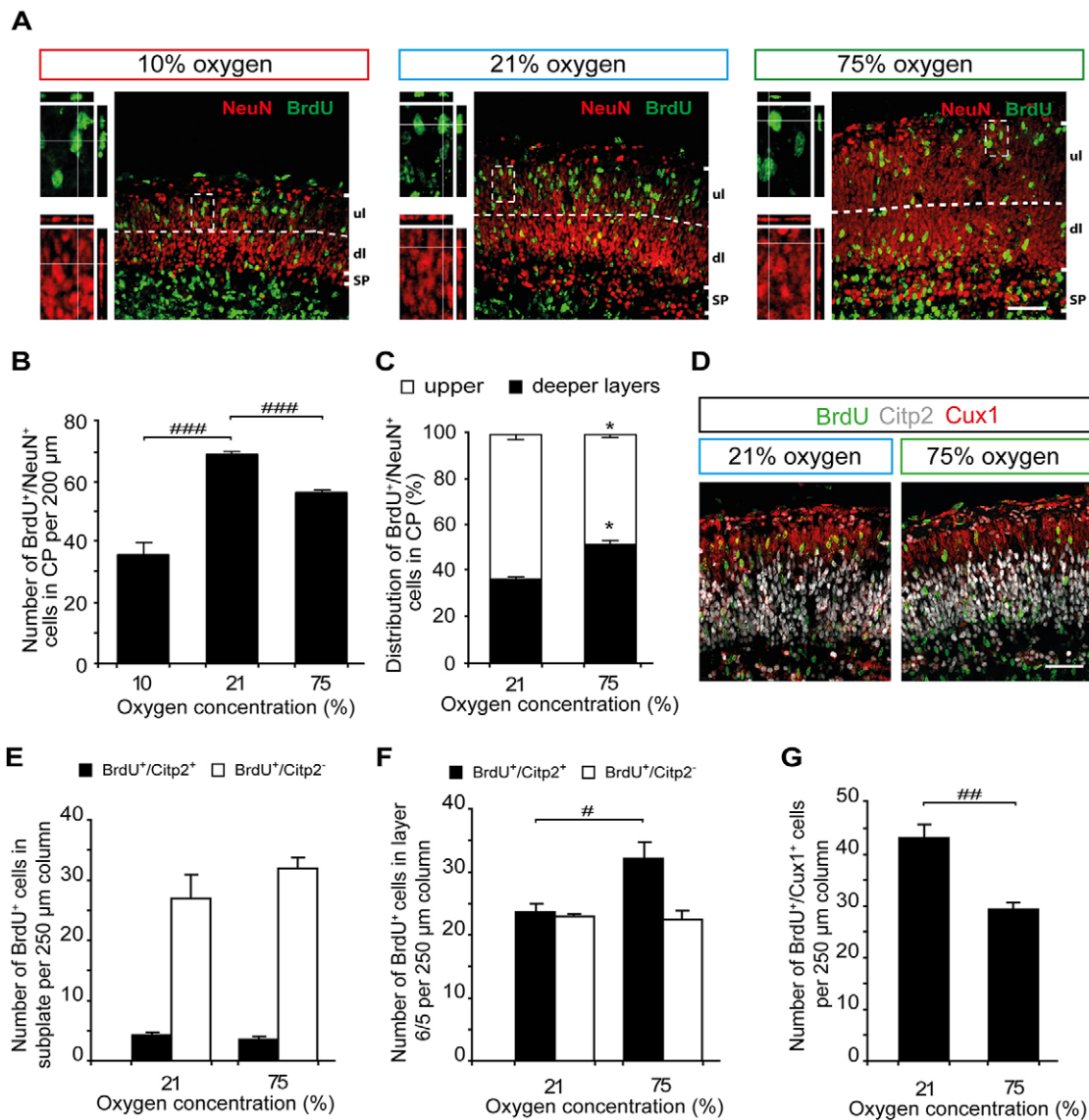
Normal development of the fetal brain can be affected by several factors, including genetic background, inflammatory insults, as well

as changes in extrinsic and intrinsic signals (Carpentier et al., 2013; Haydar et al., 1996). It emerges that oxygen signaling in neuroprogenitors appears to be a crucial mechanism for cell survival during brain development. Consistently, *in vitro* studies showed that a reduction of the atmospheric oxygen concentration from 21% to 3% promotes proliferation and stem cell maintenance of isolated neural progenitor cells (Storch et al., 2001; Studer et al., 2000). Detailed analysis of the molecular mechanism underlying the oxygen sensitivity of neuroprogenitors showed that  $Hif1\alpha$  acts as a positive regulator of the survival, growth and differentiation of NPCs *in vitro* and *in vivo* (Milosevic et al., 2007; Tomita et al., 2003; Zhao et al., 2008; Mazumdar et al., 2010). To assess whether  $Hif1\alpha$  plays an additional role in cortical neurogenesis, we used a CKO model to inactivate  $Hif1\alpha$  within neural progenitor cells. In our set of investigations,  $Hif1\alpha$  proved to be an essential factor only for angiogenesis, but did not directly regulate any feature of progenitor subtypes.

Changes in corticogenesis *in vivo* can be elicited by several mechanisms controlling fetal brain development, such as alterations in proliferation, cell survival, differentiation and migration (Marín and Rubenstein, 2003; Molyneaux et al., 2007). To understand exactly how oxygen acts in this orchestrated process, we examined the role of molecular oxygen in cortical brain development. We observed that the reduction of atmospheric oxygen to 10% for the mothers led to a distinct increase in hypoxic tissue and apoptosis in the forebrain of their offspring, which resulted in a significantly reduced brain volume and cortical thickness. We could furthermore show that the proliferation of the basal progenitors and the subsequent generation of upper cortical neurons were both downregulated by the lower oxygen tension within the fetal brain. It is also widely known that physiological oxygen levels in the mouse brain range from as low as 1% to 8% and are thus considerably lower than the inhaled ambient oxygen tension of 21% (Zhu et al., 2005). There appears to be a threshold of beneficial hypoxia, below which progenitor proliferation and neurogenesis are not improved but hampered due to increased cell death in the several germinal layers. We assume that the adaptation of physiological oxygenation to hypoxia and reoxygenation induces oxidative stress in the cells and leads to increased generation of reactive oxygen species, which damage the cells. In contrast to the *in vivo* systems, oxygen concentration within cell culture medium can be adjusted exactly so that cells are cultivated under relatively stable oxygen conditions with normal oxidative stress.

During our initial investigations of brain morphology, we could highlight that maternal hyperoxia led to greater brain volume at constant vessel density. The enlargement of the human cerebral cortex is elicited by a new specialized precursor pool, the OSVZ progenitors (Nonaka-Kinoshita et al., 2013; Stahl et al., 2013). Recent studies provided evidence that OSVZ progenitors reside not only in gyrencephalic brains of primate species or ferret, but also in low abundance in lissencephalic mammals such as rats and mice. However, owing to their poor proliferation, they do not constitute a distinct layer (Shitamukai et al., 2011; Wang et al., 2011). It is known that interspecies diversity in brain size occurs due to differences in cell cycle duration and the length of the neurogenic period affecting the abundance of the various progenitor populations (VZ, SVZ and OSVZ progenitors). Analysis of the neurogenic zones within hyperoxic mouse neocortex revealed an accumulation of proliferative progenitor cells in the superficial SVZ, forming a third separate neurogenic zone (Fig. 7). Furthermore, we found an apparent expansion of  $\text{Sox2}^{+}$  cells, as well as  $\text{Sox2}^{+}/Tbr2^{+}$  neuroprogenitors, in the IZ after hyperoxic treatment in comparison





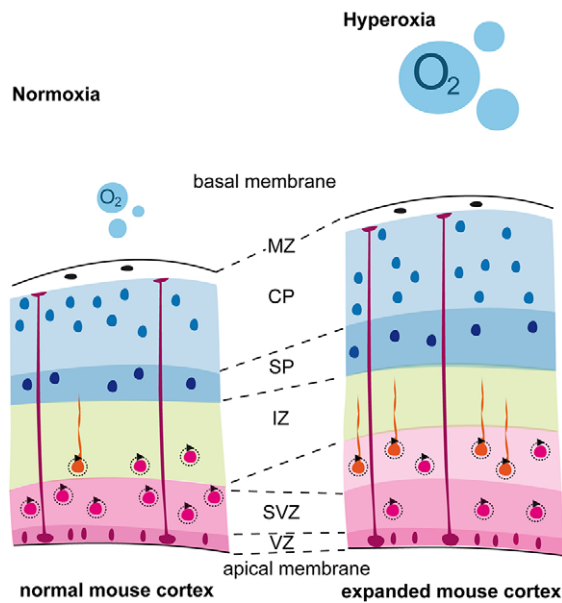
**Fig. 6. Hyperoxia affects the correct positioning of cortical neurons within the fetal brain.** (A) Representative fluorescence microscopy images showing coronal cryosections of the anterior telencephalon from different oxygen-treated littermates at E16 immunostained with BrdU and counterstained for the neuronal marker NeuN. Cortical neurons generated at E14 were labeled by BrdU. (B) Quantification of BrdU<sup>+</sup>/NeuN<sup>+</sup> cell numbers in the CP in 200 μm-wide sampling boxes of dorsal telencephalon. One-way ANOVA revealed significant differences between atmospheric oxygen tensions ( $F$ -value=68.8). (C) Distribution of BrdU<sup>+</sup>/NeuN<sup>+</sup> cells within the CP after maternal hyperoxia. (D) Representative images of normoxic and hyperoxic normal cortices stained for the deeper layer marker Ctip2 (white), upper layer marker Cux1 (red), and BrdU (green). (E) The number of BrdU<sup>+</sup>/Ctip2<sup>+</sup> and BrdU<sup>+</sup>/Ctip2<sup>-</sup> cells in the SP in 250 μm-wide sampling boxes. Statistical analysis revealed no significant differences between both oxygen tensions ( $P \geq 0.05$ , unpaired Student's  $t$ -test). (F) Number of BrdU<sup>+</sup>/Ctip2<sup>+</sup> and BrdU<sup>+</sup>/Ctip2<sup>-</sup> cells in layer 6/5 in 250 μm-wide sampling boxes. There were significant differences in the number of BrdU<sup>+</sup>/Ctip2<sup>+</sup> cells between both oxygen tensions ( $P=0.017$ , unpaired Student's  $t$ -test). (G) Quantification of BrdU<sup>+</sup>/Cux1<sup>+</sup> cells in the upper layers in 250 μm-wide sampling boxes. There were significant differences between both oxygen tensions ( $P=0.006$ , unpaired Student's  $t$ -test). Data are mean  $\pm$  s.e.m.  $n=4$ . # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$ ; \* $P < 0.05$ , when compared with 21% oxygen tension. dl, deep layers; ul, upper layers. Scale bars: 50 μm.

to the normoxic samples. Since we showed that in the hyperoxic IZ more cells possessed the characteristic p-Vim<sup>+</sup> basal process of OSVZ progenitors (Wang et al., 2011), it is very unlikely that these cells are simply displaced RGCs. Moreover, marker expression analysis of these cells revealed that they express Sox2<sup>+</sup>/Tbr2<sup>+</sup>, indicating that they have a radial glia origin (Betizeau et al., 2013).

Our results demonstrate further that under hyperoxia the region basal to the SVZ contains large numbers of mitotic progenitors and becomes the predominant proliferative zone at the expense of SVZ progenitor proliferation during mid-gestation, now constituting a distinct layer (Fig. 7). Interestingly, the total proliferation rate of all neurogenic regions was not altered between normoxic and

hyperoxic forebrains but the number of neuroprogenitors in the SVZ and IZ was significantly increased after maternal hyperoxia, indicating that the accumulation of these cells is likely to be caused by self-amplification of existing OSVZ-like progenitors within the IZ. Based on these findings, we speculate that higher tissue oxygen tension during this time frame promotes the proliferation of the rare population of OSVZ-like progenitors in the IZ of the rodent brain.

To prove the temporally specific effects of increased oxygen tension on neurogenic layer development, we elucidated the impact of oxygen at early stages of mammalian neurogenesis in order to define its regulatory role in SVZ neurogenesis. We did not observe



**Fig. 7. Schematic of the expansion of the neocortex in response to hyperoxia in mouse.** In normoxic fetal mouse cortex (left) the ventricular zone (VZ) and subventricular zone (SVZ) produce most of the neurons, whereas the intermediate zone (IZ) contains only very few proliferating cells. Increased cortical oxygen tension (hyperoxia, right) leads to an increase in proliferative cells with OSVZ-like morphology in the IZ, leading to the formation of a new proliferative cell layer, while SVZ progenitor proliferation decreases. The generation of upper cortical layer neurons is diminished, while more newborn neurons head for deeper layers, as a part of the cortical plate (CP). MZ, marginal zone; SP, subplate.

any differences in SVZ Tbr2<sup>+</sup>/pH3<sup>+</sup> progenitor proliferation and no alterations in the number of intermediate Tbr2<sup>+</sup> progenitors upon hyperoxic treatment at E10-12. Although we confirmed that E12 brains show a similar pattern of hypoxic tissue as E16 brains, we did not detect any changes in tissue oxygen tension in the fetal brain after maternal hyperoxic treatment with 75% atmospheric oxygen for 48 h, which is most likely due to the near complete absence of brain vascularization at this developmental stage (Chen et al., 1999; Lee et al., 2001; Simon and Keith, 2008). Higher or longer oxygen administrations were not tolerated by the adult animals. We are thus unable to resolve whether the effects of hyperoxia on OSVZ-like progenitors are specific to the developmental stage or whether the absence of any effects of oxygen on SVZ development is related to very stable brain oxygen tensions even after maximal tolerated maternal oxygen treatment.

Cell survival was enhanced in hyperoxia in comparison to normoxic brains, suggesting that the accumulated progenitors within the IZ could contribute to cortical neurogenesis. Evaluation of the various cortical identities revealed an increase in lower layer neurons. To establish whether this effect resulted from a higher rate of neurogenesis, we performed a BrdU birth-dating study. We found that hyperoxia during mid-gestation did not affect the total number of newly generated neurons, indicating no disturbance in proliferation. Furthermore, the integrated cortical neurons were located mainly in the deeper layers of the hyperoxic cortex (Fig. 7), whereas they should normally reside in the upper layers of the cortex as in normoxic brains. Interestingly, we found a reduction of mitotic cells within the SVZ, which represents the main source of all cortical neurons (Kowalczyk et al., 2009; Vasistha et al., 2014). However, the total number of integrated cortical neurons was not altered, indicating that the progenitor cells within

the IZ contribute to corticogenesis and apparently generate cortical neurons during maternal hyperoxia, which were specified for deeper cortical layers. Indeed, OSVZ progenitors produce subgranular layer neurons in the primate brain (layers I-IV) (Lukaszewicz et al., 2005), but no distinct evidence of their commitment in rodent corticogenesis has existed until now. We thus speculate that the expansion of mitotic OSVZ-like cells and simultaneous increase in deeper cortical layer cells indicate that these progenitors actively contribute to cortical neurogenesis and that the IPs generated from these cells are fated to form deeper layer neurons, thereby endowing the brain with higher complexity. However, we cannot rule out the possibility that the newborn neurons are also generated by apical progenitor cells or prematurely end their migration as a result of cortical hyperoxia.

In conclusion, our data highlight that oxygen signaling in neuroprogenitors is an essential mechanism during mid-neurogenesis, especially for cell proliferation and cell commitment in regions basal to the SVZ, and therefore pivotal for the neurogenesis of cortical neurons. Our observations indeed led to the hypothesis that oxygen tension may initiate the expansion of the OSVZ-like cells by inducing self-renewing divisions, which actively participate in cortical neurogenesis to augment brain complexity. These findings might lead in the future to the establishment of pre-emptive therapeutic strategies that exploit oxygen effects during gestation in the case of maternal illness, allergy or asthma, which, accompanied with transient fetal hypoxia, might prevent the development of neurological conditions such as autism spectrum disorder or schizophrenia in offspring. Moreover, better insights into the signaling cascades involved might lead to new approaches for the reactivation of non-neurogenic, Sox2<sup>+</sup> OSVZ progenitor cells in the postnatal cortex.

## MATERIALS AND METHODS

### Animals and genotyping

Mice were maintained on a C57BL/6 background. All animal protocols were approved by the local animal welfare legislation. We removed Hif1 $\alpha$  in mouse embryos using a conditional mutation created by a Nestin-Cre strain to specifically ablate Hif1 $\alpha$  in all neural progenitors of the embryonic brain and crossed them with *Hif1a*<sup>fl $\alpha$ /fl $\alpha$</sup>  mice, in which exons 13-15 of *Hif1a* are flanked by loxP sites (Tomita et al., 2003). *Hif1a*<sup>fl $\alpha$ /fl $\alpha$</sup>  littermates served as controls (normal condition) to reduce differences related to housing in the closed oxygen chamber and to the oxygen supply of fetuses as a result of maternal factors such as placenta capacity. In initial experiments, no differences in hypoxic brain area, brain size and cortical development were observed in mice of *Hif1a*<sup>fl $\alpha$ /fl $\alpha$</sup>  genotype as compared with wild-type mice. For further details, see the supplementary Materials and Methods.

### Oxygen treatment

The experimental setup is schematized in Fig. 1A. E14 timed-pregnant Tomita mice were exposed to 10% or 75% oxygen in a closed oxygen chamber (InerTec) for 48 h. Normoxic mice were incubated in room air. To investigate the role of brain tissue oxygen tension at earlier stages of cortical neurogenesis (SVZ development), brains of normal and *Hif1a* CKO litters were examined after exposing pregnant mice to normoxic (21%) or hyperoxic (75%) conditions from E10 to E12, similar to the approach described above. For further details, see the supplementary Materials and Methods.

### Birth-dating study and immunohistochemistry

For analysis of the influence of oxygen on the generation of cortical neurons and their migration to the CP, E14 pregnant mice received intraperitoneal injections of 50 mg/kg 5-bromo-2'-deoxyuridine (BrdU; SERVA Electrophoresis) in 0.9% NaCl once before oxygen treatment. The embryos were dissected after 48 h, and their fixed brains were frozen and

coronally sectioned and immunostained according to standard protocols. For further details, including the TUNEL assay, see the supplementary Materials and Methods.

### Image acquisition and quantitative measurements

Images for quantification were captured using an Axio Observer.Z1 spinning disc confocal microscope (Zeiss) and optimized for brightness and contrast using Fiji (NIH). The imaging parameters were kept constant during imaging. Details are provided in the supplementary Materials and Methods.

### Statistical analysis

Statistical analyses were performed with IBM SPSS Statistic software (version 20.0). Data are represented as mean±s.e.m. Statistical significance was determined by two-way and one-way ANOVA with the Bonferroni's post-hoc *t*-test as appropriate. All experiments were independently replicated at least four times. *P*<0.05 (two-tailed test) was considered significant.

### Acknowledgements

We thank Sylvia Kanzler, Andrea Kempe and Cornelia May for technical help; and Shuhei Tomita, MD, PhD for generously providing *Hif1a* floxed mice.

### Competing interests

The authors declare no competing or financial interests.

### Author contributions

L.W. and A.K.M.: conception and design, collection and assembly of data, data analysis and interpretation, drafting and revision of manuscript. L.B. and L.M.: data analysis and interpretation, drafting and revision of manuscript. A.S.: conception and design, data analysis and interpretation, drafting and revision of manuscript, and fund raising.

### Funding

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) through the Collaborative Research Center [SFB 655] 'Cells into Tissues' (project A23) to A.S.

### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.121939/-DC1>

### References

- Alcamo, E. A., Chirivella, L., Dautzenberg, M., Dobрева, G., Fariñas, I., Grosschedl, R. and McConnell, S. K. (2008). *Satb2* regulates callosal projection neuron identity in the developing cerebral cortex. *Neuron* **57**, 364-377.
- Arlotta, P., Molyneaux, B. J., Chen, J., Inoue, J., Kominami, R. and Macklis, J. D. (2005). Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. *Neuron* **45**, 207-221.
- Betizeau, M., Cortay, V., Patti, D., Pfister, S., Gautier, E., Bellemin-Ménard, A., Afanassieff, M., Huissoud, C., Douglas, R. J., Kennedy, H. et al. (2013). Precursor diversity and complexity of lineage relationships in the outer subventricular zone of the primate. *Neuron* **80**, 442-457.
- Biaschke, A. J., Staley, K. and Chun, J. (1996). Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. *Development* **122**, 1165-1174.
- Borrell, V. and Reillo, I. (2012). Emerging roles of neural stem cells in cerebral cortex development and evolution. *Dev. Neurobiol.* **72**, 955-971.
- Bruick, R. K. (2003). Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. *Genes Dev.* **17**, 2614-2623.
- Bulfone, A., Martinez, S., Marigo, V., Campanella, M., Basile, A., Quaderi, N., Gattuso, C., Rubenstein, J. L. R. and Ballabio, A. (1999). Expression pattern of the *Tbr2* (Eomesodermin) gene during mouse and chick brain development. *Mech. Dev.* **84**, 133-138.
- Carpentier, P. A., Haditsch, U., Braun, A. E., Cantu, A. V., Moon, H. M., Price, R. O., Anderson, M. P., Saravanapandian, V., Ismail, K., Rivera, M. et al. (2013). Stereotypical alterations in cortical patterning are associated with maternal illness-induced placental dysfunction. *J. Neurosci.* **33**, 16874-16888.
- Chen, E. Y., Fujinaga, M. and Giaccia, A. J. (1999). Hypoxic microenvironment within an embryo induces apoptosis and is essential for proper morphological development. *Teratology* **60**, 215-225.
- Chenn, A. and Walsh, C. A. (2002). Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* **297**, 365-369.
- Davies, P. W. and Brink, F. (1942). *Microelectrodes for Measuring Local Oxygen Tension in Animal Tissues*. Johnson Research Foundation: Philadelphia.
- Dehay, C. and Kennedy, H. (2007). Cell-cycle control and cortical development. *Nat. Rev. Neurosci.* **8**, 438-450.
- Englund, C., Fink, A., Lau, C., Pham, D., Daza, R. A. M., Bulfone, A., Kowalczyk, T. and Hevner, R. F. (2005). *Pax6*, *Tbr2*, and *Tbr1* are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J. Neurosci.* **25**, 247-251.
- Fietz, S. A., Kelava, I., Vogt, J., Wilsch-Bräuninger, M., Stenzel, D., Fish, J. L., Corbeil, D., Riehn, A., Distler, W., Nitsch, R. et al. (2010). OSVZ progenitors of human and ferret neocortex are epithelial-like and expand by integrin signaling. *Nat. Neurosci.* **13**, 690-699.
- Fish, J. L., Dehay, C., Kennedy, H. and Huttner, W. B. (2008). Making bigger brains—the evolution of neural-progenitor-cell division. *J. Cell Sci.* **121**, 2783-2793.
- Florio, M. and Huttner, W. B. (2014). Neural progenitors, neurogenesis and the evolution of the neocortex. *Development* **141**, 2182-2194.
- Franco, S. J., Gil-Sanz, C., Martinez-Garay, I., Espinosa, A., Harkins-Perry, S. R., Ramos, C. and Muller, U. (2012). Fate-restricted neural progenitors in the mammalian cerebral cortex. *Science* **337**, 746-749.
- Frantz, G. D. and McConnell, S. K. (1996). Restriction of late cerebral cortical progenitors to an upper-layer fate. *Neuron* **17**, 55-61.
- Gal, J. S., Morozov, Y. M., Ayoub, A. E., Chatterjee, M., Rakic, P. and Haydar, T. F. (2006). Molecular and morphological heterogeneity of neural precursors in the mouse neocortical proliferative zones. *J. Neurosci.* **26**, 1045-1056.
- Hansen, D. V., Lui, J. H., Parker, P. R. L. and Kriegstein, A. R. (2010). Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nature* **464**, 554-561.
- Hashimoto, T. and Shibasaki, F. (2015). Hypoxia-inducible factor as an angiogenic master switch. *Front. Pediatr.* **3**, 33.
- Haubensak, W., Attardo, A., Denk, W. and Huttner, W. B. (2004). From The Cover: Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc. Natl. Acad. Sci. USA* **101**, 3196-3201.
- Haydar, T. F., Blue, M. E., Molliver, M. E., Krueger, B. K. and Yarowsky, P. J. (1996). Consequences of trisomy 16 for mouse brain development: corticogenesis in a model of Down syndrome. *J. Neurosci.* **16**, 6175-6182.
- Haydar, T. F., Kuan, C.-Y., Flavell, R. A. and Rakic, P. (1999). The role of cell death in regulating the size and shape of the mammalian forebrain. *Cereb. Cortex* **9**, 621-626.
- Hevner, R. F., Shi, L., Justice, N., Hsueh, Y.-P., Sheng, M., Smiga, S., Bulfone, A., Goffinet, A. M., Campagnoni, A. T. and Rubenstein, J. L. (2001). *Tbr1* regulates differentiation of the preplate and layer 6. *Neuron* **29**, 353-366.
- Hutton, S. R. and Pevny, L. H. (2011). SOX2 expression levels distinguish between neural progenitor populations of the developing dorsal telencephalon. *Dev. Biol.* **352**, 40-47.
- Insolera, R., Bazzi, H., Shao, W., Anderson, K. V. and Shi, S.-H. (2014). Cortical neurogenesis in the absence of centrioles. *Nat. Neurosci.* **17**, 1528-1535.
- Ivanovic, Z. (2009). Hypoxia or in situ normoxia: the stem cell paradigm. *J. Cell. Physiol.* **219**, 271-275.
- Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y. et al. (1998). Cellular and developmental control of O<sub>2</sub> homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev.* **12**, 149-162.
- Kaidi, A., Williams, A. C. and Paraskeva, C. (2007). Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nat. Cell Biol.* **9**, 210-217.
- Kamei, Y., Inagaki, N., Nishizawa, M., Tsutsumi, O., Taketani, Y. and Inagaki, M. (1998). Visualization of mitotic radial glial lineage cells in the developing rat brain by Cdc2 kinase-phosphorylated vimentin. *Glia* **23**, 191-199.
- Kowalczyk, T., Pontious, A., Englund, C., Daza, R. A. M., Bedogni, F., Hodge, R., Attardo, A., Bell, C., Huttner, W. B. and Hevner, R. F. (2009). Intermediate neuronal progenitors (basal progenitors) produce pyramidal-projection neurons for all layers of cerebral cortex. *Cereb. Cortex* **19**, 2439-2450.
- Kriegstein, A. and Alvarez-Buylla, A. (2009). The glial nature of embryonic and adult neural stem cells. *Annu. Rev. Neurosci.* **32**, 149-184.
- Kriegstein, A., Noctor, S. and Martínez-Cerdeño, V. (2006). Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. *Nat. Rev. Neurosci.* **7**, 883-890.
- Lee, Y. M., Jeong, C.-H., Koo, S.-Y., Son, M. J., Song, H. S., Bae, S.-K., Raleigh, J. A., Chung, H.-Y., Yoo, M.-A. and Kim, K.-W. (2001). Determination of hypoxic region by hypoxia marker in developing mouse embryos in vivo: a possible signal for vessel development. *Dev. Dyn.* **220**, 175-186.
- Lukaszewicz, A., Savatier, P., Cortay, V., Giroud, P., Huissoud, C., Berland, M., Kennedy, H. and Dehay, C. (2005). G1 phase regulation, area-specific cell cycle control, and cytoarchitectonics in the primate cortex. *Neuron* **47**, 353-364.
- Ma, S., Kwon, H. J., Johng, H., Zang, K. and Huang, Z. (2013). Radial glial neural progenitors regulate nascent brain vascular network stabilization via inhibition of Wnt signaling. *PLoS Biol.* **11**, e1001469.
- Malatesta, P., Hartfuss, E. and Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* **127**, 5253-5263.



- Marín, O. and Rubenstein, J. L.** (2003). Cell migration in the forebrain. *Annu. Rev. Neurosci.* **26**, 441-483.
- Marin-Padilla, M.** (1978). Dual origin of the mammalian neocortex and evolution of the cortical plate. *Anat. Embryol. (Berl.)* **152**, 109-126.
- Mazumdar, J., O'Brien, W. T., Johnson, R. S., LaManna, J. C., Chavez, J. C., Klein, P. S. and Simon, M. C.** (2010). O<sub>2</sub> regulates stem cells through Wnt/beta-catenin signalling. *Nat. Cell Biol.* **12**, 1007-1013.
- Milosevic, J., Maisel, M., Wegner, F., Leuchtenberger, J., Wenger, R. H., Gerlach, M., Storch, A. and Schwarz, J.** (2007). Lack of hypoxia-inducible factor-1 alpha impairs midbrain neural precursor cells involving vascular endothelial growth factor signaling. *J. Neurosci.* **27**, 412-421.
- Miyata, T., Kawaguchi, A., Okano, H. and Ogawa, M.** (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* **31**, 727-741.
- Miyata, T., Kawaguchi, A., Saito, K., Kawano, M., Muto, T. and Ogawa, M.** (2004). Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development* **131**, 3133-3145.
- Mohyeldin, A., Garzón-Muvdi, T. and Quiñones-Hinojosa, A.** (2010). Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell* **7**, 150-161.
- Molnár, Z., Métin, C., Stoykova, A., Tarabykin, V., Price, D. J., Francis, F., Meyer, G., Dehay, C. and Kennedy, H.** (2006). Comparative aspects of cerebral cortical development. *Eur. J. Neurosci.* **23**, 921-934.
- Molyneaux, B. J., Arlotta, P., Menezes, J. R. L. and Macklis, J. D.** (2007). Neuronal subtype specification in the cerebral cortex. *Nat. Rev. Neurosci.* **8**, 427-437.
- Nieto, M., Monuki, E. S., Tang, H., Imitola, J., Haubst, N., Khoury, S. J., Cunningham, J., Gotz, M. and Walsh, C. A.** (2004). Expression of Cux-1 and Cux-2 in the subventricular zone and upper layers II-IV of the cerebral cortex. *J. Comp. Neurol.* **479**, 168-180.
- Noctor, S. C., Flint, A. C., Weissman, T. A., Dammernan, R. S. and Kriegstein, A. R.** (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409**, 714-720.
- Noctor, S. C., Martínez-Cerdeño, V., Ivic, L. and Kriegstein, A. R.** (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.* **7**, 136-144.
- Nonaka-Kinoshita, M., Reillo, I., Artegiani, B., Martínez-Martínez, M. Á., Nelson, M., Borrell, V. and Calegari, F.** (2013). Regulation of cerebral cortex size and folding by expansion of basal progenitors. *EMBO J.* **32**, 1817-1828.
- Parnavelas, J. G.** (2000). The origin and migration of cortical neurones: new vistas. *Trends Neurosci.* **23**, 126-131.
- Price, D. J., Aslam, S., Tasker, L. and Gillies, K.** (1997). Fates of the earliest generated cells in the developing murine neocortex. *J. Comp. Neurol.* **377**, 414-422.
- Raleigh, J. A., Miller, G. G., Franko, A. J., Koch, C. J., Fuciarelli, A. F. and Kelly, D. A.** (1987). Fluorescence immunohistochemical detection of hypoxic cells in spheroids and tumours. *Br. J. Cancer* **56**, 395-400.
- Reillo, I., de Juan Romero, C., Garcia-Cabezas, M. A. and Borrell, V.** (2011). A role for intermediate radial glia in the tangential expansion of the mammalian cerebral cortex. *Cereb. Cortex* **21**, 1674-1694.
- Roy, K., Kuznicki, K., Wu, Q., Sun, Z., Bock, D., Schutz, G., Vranich, N. and Monaghan, A. P.** (2004). The Tlx gene regulates the timing of neurogenesis in the cortex. *J. Neurosci.* **24**, 8333-8345.
- Semenza, G. L.** (1999). Regulation of mammalian O<sub>2</sub> homeostasis by hypoxia-inducible factor 1. *Annu. Rev. Cell Dev. Biol.* **15**, 551-578.
- Semenza, G. L.** (2006). Regulation of physiological responses to continuous and intermittent hypoxia by hypoxia-inducible factor 1. *Exp. Physiol.* **91**, 803-806.
- Seylaz, J. and Pinard, E.** (1978). Continuous measurement of gas partial pressures in intracerebral tissue. *J. Appl. Physiol. Environ. Exerc. Physiol.* **44**, 528-533.
- Sharp, F. R. and Bernaudin, M.** (2004). HIF1 and oxygen sensing in the brain. *Nat. Rev. Neurosci.* **5**, 437-448.
- Shen, Q., Wang, Y., Dimos, J. T., Fasano, C. A., Phoenix, T. N., Lemischka, I. R., Ivanova, N. B., Stifani, S., Morrisey, E. E. and Temple, S.** (2006). The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. *Nat. Neurosci.* **9**, 743-751.
- Sheppard, A. M. and Pearlman, A. L.** (1997). Abnormal reorganization of preplate neurons and their associated extracellular matrix: an early manifestation of altered neocortical development in the reeler mutant mouse. *J. Comp. Neurol.* **378**, 173-179.
- Shitamukai, A., Konno, D. and Matsuzaki, F.** (2011). Oblique radial glial divisions in the developing mouse neocortex induce self-renewing progenitors outside the germinal zone that resemble primate outer subventricular zone progenitors. *J. Neurosci.* **31**, 3683-3695.
- Simon, M. C. and Keith, B.** (2008). The role of oxygen availability in embryonic development and stem cell function. *Nat. Rev. Mol. Cell Biol.* **9**, 285-296.
- Smart, I. H. M., Dehay, C., Giroud, P., Berland, M. and Kennedy, H.** (2002). Unique morphological features of the proliferative zones and postmitotic compartments of the neural epithelium giving rise to striate and extrastriate cortex in the monkey. *Cereb. Cortex* **12**, 37-53.
- Stahl, R., Walcher, T., De Juan Romero, C., Pilz, G. A., Cappello, S., Irmiler, M., Sanz-Aguela, J. M., Beckers, J., Blum, R., Borrell, V. et al.** (2013). Tmp1 regulates expansion and folding of the mammalian cerebral cortex by control of radial glial fate. *Cell* **153**, 535-549.
- Storch, A., Paul, G., Csete, M., Boehm, B. O., Carvey, P. M., Kupsch, A. and Schwarz, J.** (2001). Long-term proliferation and dopaminergic differentiation of human mesencephalic neural precursor cells. *Exp. Neurol.* **170**, 317-325.
- Studer, L., Csete, M., Lee, S. H., Kabbani, N., Walikonis, J., Wold, B. and McKay, R.** (2000). Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. *J. Neurosci.* **20**, 7377-7383.
- Tomita, S., Ueno, M., Sakamoto, M., Kitahama, Y., Ueki, M., Maekawa, N., Sakamoto, H., Gassmann, M., Kageyama, R., Ueda, N. et al.** (2003). Defective brain development in mice lacking the Hif-1alpha gene in neural cells. *Mol. Cell. Biol.* **23**, 6739-6749.
- Vasistha, N. A., García-Moreno, F., Arora, S., Cheung, A. F. P., Arnold, S. J., Robertson, E. J. and Molnár, Z.** (2014). Cortical and clonal contribution of Tbr2 expressing progenitors in the developing mouse brain. *Cereb. Cortex*, doi: 10.1093/cercor/bhu125.
- Wang, X., Tsai, J.-W., LaMonica, B. and Kriegstein, A. R.** (2011). A new subtype of progenitor cell in the mouse embryonic neocortex. *Nat. Neurosci.* **14**, 555-561.
- Zhao, T., Zhang, C.-p., Liu, Z.-h., Wu, L.-y., Huang, X., Wu, H.-t., Xiong, L., Wang, X., Wang, X.-m., Zhu, L.-l. et al.** (2008). Hypoxia-driven proliferation of embryonic neural stem/progenitor cells - role of hypoxia-inducible transcription factor-1alpha. *FEBS J.* **275**, 1824-1834.
- Zhu, L.-L., Wu, L.-Y., Yew, D. T. and Fan, M.** (2005). Effects of hypoxia on the proliferation and differentiation of NSCs. *Mol. Neurobiol.* **31**, 231-242.